Environmental Health Perspectives
Vol. 100, pp. 307–315, 1993

Genetic Toxicology: Current Status of Methods of Carcinogen Identification

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A critical aspect of the efforts to relate the results of short-term genetic toxicity tests with those from long-term rodent tests for carcinogens is the quality and consistency of the studies conducted by the National Toxicology Program. Analysis of the results in relationship to chemical structure has shown that mutagenic potential is a primary risk factor for carcinogen identification. Chemicals positive in the Salmonella assay generally possess "structural alerts" for electrophilic interactions, are predominantly represented among chemicals producing *trans*-species carcinogenic effects in rodents, and among those identified as carcinogenic to humans. Current efforts are aimed at defining toxicological, structural, and mechanistic properties of nonmutagens that are carcinogenic in rodents.

Introduction

Genetic toxicology came into existence as a separate discipline in the mid-1960s. The origin of the field was tied to a growing understanding of the mutagenic basis of some cancers and a strong association between radiation, induced mutation, and cancer. Development of the field was also motivated by concerns for the effects of chemicals and environmental factors on the human gene pool and the transmission of induced mutations to subsequent generations. Over a period of approximately 3 decades, the discipline has evolved significantly and has contributed to a clearer understanding of relationships among chemical structure, induced mutagenesis, and cancer. The contributions of genetic toxicology to understanding the role of induced mutations in heritable diseases is covered in another article in this issue by Shelby et al. (*1*). In this paper we summarize information derived principally from chemicals studied under the aegis of the National Toxicology Program (NTP) and describe relationships between chemical structures and biological effects related to cancer. We also discuss *in vitro* and short-term *in vivo* methods used for identifying potential mutagens and carcinogens and their value and limitations. In addition, current efforts to improve methods of carcinogen identification and to understand the properties of carcinogenic chemicals that are not mutagenic are summarized.

Assay Selection and Validation

The concept of "validation" of an assay implies that the measured end point is truly a characteristic of the system and that the results of the assay are reproducible within and between laboratories. Various approaches and solutions to the problems of validation have been proposed over the past two decades and have often involved the use of chemicals of known carcinogenic potential. Such chemicals were used to demonstrate that a proposed assay system could detect or identify a majority of such carcinogens (defined as "sensitivity"). An adequate validation study also involves the accurate identification of chemicals that are known to be noncarcinogens. Such validation of "specificity" is equally important for establishing the reliability of the assay. The database generated by the National Toxicology Program contains equivalent numbers of rodent carcinogens and noncarcinogens.

Approximately 5–10 years ago there were clear indications from the NTP studies that the strong relationship that had been proposed between mutagenicity and rodent carcinogenicity, as defined by data derived from the Ames Salmonella mutagenesis test (*2,3*), had some deficiencies. Many chemicals were carcinogenic to rodents but were not identified as mutagens by the Salmonella test (*4–6*). These observations led to extensive efforts to search for other *in vitro* or short-term *in vivo* methods that would complement the Salmonella test and lead to methods that were uniformly predictive for carcinogenic potential. However, an evaluation of published reports from these systems, conducted by the U.S. Environmental Protection Agency (EPA) Gene Tox Program, revealed that there were major impediments to reaching any conclusions about the capacity of these assays to predict carcinogens. First, inadequate numbers of chemicals had been tested in common across the various assay systems to permit any form of statistical comparisons. That is, although some assays had been used to test a large number of chemicals, few of the chemicals were in common with those tested in other assay systems. Second, the database contained inadequate numbers of noncarcinogenic chemicals. For example, the EPA Gene-Tox compilation of chemicals tested in Salmonella contained only 4.4% noncarcinogens (*7*). Third, the data were often of inconsistent quality, both within and among assay systems. Little

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effort had been made to standardize assays, and there was a great deal of uncertainty about which variables in the protocol and test methodology produced the major sources of variation.

Test systems were initially selected for use in the NTP genetic toxicology testing program based on their widespread use and the extent to which the genetics of the organism was understood. It was determined at the outset of the program's development that only a limited number of tests were sufficiently well developed and used to provide information on the ability of a chemical to induce gene mutations and chromosome damage. The tests initially selected were the *Salmonella typhimurium*/mammalian microsome (Ames) test: measurement of thymidine kinase resistance in cultured mouse lymphoma L5178Y cells; induction of chromosome aberrations and sister chromatid exchanges in cultured Chinese hamster ovary cells; and induction of sex-linked recessive lethal mutations and reciprocal translocations in the fruit fly, *Drosophila melanogaster* (*8*). Later in the development of the program, a number of *in vivo* cytogenetic tests was added. These were the measurement of chromosome aberrations and sister chromatid exchanges in mouse bone marrow; the measurement of micronuclei in mouse and rat bone marrow, and the measurement of micronuclei in peripheral blood erythrocytes of mice.

To be of value in decision making, and to meet the rigorous demands of scientific validity, mutagenicity test results must be reproducible both qualitatively and quantitatively. The validation procedures used entailed the testing of selected chemicals to determine if the test truly measured what it was designed to measure; assessing the intra- and interlaboratory reproducibility of the tests; and development of standardized protocols (*8,9*). Validation studies were performed with a number of *in vitro* and *in vivo* tests (*4,10–19*). One aspect of test system development and validation was the development ot statistical procedures and approaches for determining the acceptability of the test data and for evaluating the test data (*20–27*).

Correlations with Carcinogenicity

Based on the factors discussed above, the Cellular and Genetic Toxicology Branch of the National Insitute of Environmental Health Sciences (NIEHS) conducted a large-scale objective study directed at evaluating the utility of genetic toxicity tests for identifying carcinogens and determining how the various short-term tests and other information about a chemical can be combined to yield a useful prediction of a chemical's carcinogenicity. The overall structure of the study was to choose a random group of chemicals that had recently undergone 2-year carcinogenicity bioassays in rodents. The value of such an approach was that the chemicals were not preselected on the basis of knowledge of whether they were carcinogenic, or on whether they contained particular chemical structures. Second, based on the general characteristics of the NTP database, this grouping was expected to include a sizable proportion of chemicals that failed to show carcinogenic potential when mice and rats were exposed for 104 weeks at maximum-tolerated doses. This group of noncarcinogenic substances is extremely important to the conclusions that have been derived from the studies. The definition of noncarcinogenicity is obviously operational because if the chemicals had been administered by some other route, at some other dose, or to some other rodent strains or species, there might have been a different effect. However, under the conditions of the bioassays under which many chemicals were identified as carcinogens, the chemicals that were classified as noncarcinogens failed to induce neoplasia. Chemicals showing equivocal evidence of carcinogenicity were considered noncarcinogens for the purposes of this study.

To further ensure objectivity in the evaluation process, all chemicals were tested in the genetic toxicity tests under code, using standardized protocols to remove all investigator bias. All testing was completed before the results were interpreted and the code was broken. Initially, 73 chemicals that had undergone rodent carcinogenicity bioassays were selected for testing in four *in vitro* assay systems. These assays included the Salmonella (Ames) mutagenesis assay (SAL); induction of mutations at the *tk* locus in L5178Y mouse lymphoma cells (MLA); and induction of chromosome aberrations (ABS) and sister chromatid exchanges (SCE) in Chinese hamster ovary (CHO) cells. A few of the same 73 chemicals were tested in other assays. These assays included mutagenesis in Drosophila; chromosome damage (chromosome aberrations, SCE, and micronuclei) in rodent bone marrow cells; induction of unscheduled DNA synthesis (UDS) in rodent hepatocytes; *in vitro* mammalian cell transformation; and inhibition of intercellular communication *in vitro*. The results of the initial studies using SAL, MLA, ABS, and SCE were verified and extended by an independent study of an additional 41 chemicals, bringing together a total of 114 chemicals for which both long-term effects in rodents as well as short-term effects *in vitro* have been systematically evaluated (*28*).

Some generalized characteristics of the associations between each of the four principal *in vitro* assay systems and rodent carcinogenesis are presented in Table 1. The following conclusions were reached based on the initial 73 chemicals and verified by the additional 41 chemicals. Among the four assay systems, the Salmonella assay demonstrated both the highest specificity and the lowest sensitivity. The positive predictivity of SAL was the highest (89% of the Salmonella-positive chemicals were carcinogens). Conversely the SCE and mouse lymphoma assays had the highest sensitivities (69% and 72%, respectively), but the lowest positive predictivities (64% and 63%). Thus, the Salmonella assay identifies a high proportion of chemicals with mutagenic potential that are also carcinogens, but a significant

Table 1. Operational characteristics of *in vitro* genetic tests.

	Test							
	SAL		ABS		SCE		MLA	
	+	−	+	−	+	−	+	−
Carcinogenesis								
+	32	35	35	32	46	21	48	19
−	4	43	13	34	26	21	28	19
Significance of association, *p*	<0.0001		0.007		0.105		0.127	
Sensitivity, %	48		52		69		72	
Specificity, %	91		72		45		40	
Positive predictivity, %	89		73		64		63	
Negative predictivity, %	55		52		50		50	
Concordance, %	66		61		59		59	

Abbreviations: SAL, Salmonella (Ames) mutagenesis assay; ABS, induction of chromosome aberrations; SCE, sister chromatid exchange; MLA induction of mutations at the *tk* locus in L1578Y mouse lymphoma cells.

number of carcinogens lack demonstrable mutagenic potential in this assay.

In order for a test battery to be more effective than individual tests for identifying carcinogens, the tests should complement

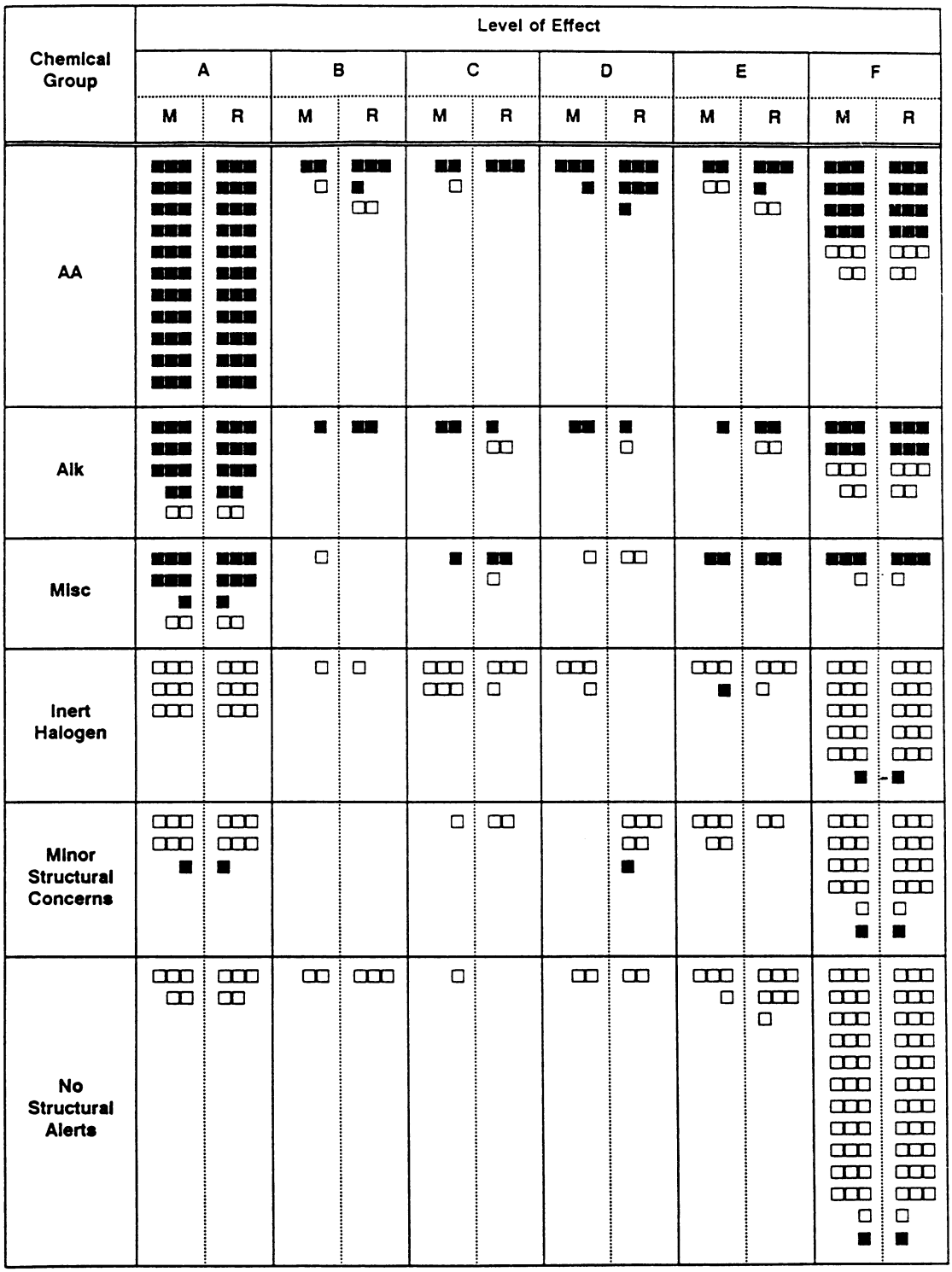

FIGURE 1. Frequency distribution of mutagens and nonmutagens as a function of chemical group and level of carcinogenicity. ■, Salmonella mutagen; □, Salmonella nonmutagen. Level of effect: A, carcinogenic in rats and mice at one or more sites; B, carcinogenic in a single species; C, carcinogenic at single site in both sexes of a single species; D, carcinogenic at a single site in a single sex of a single species; E, equivocal evidence of carcinogenicity; F, noncarcinogenic; M, mouse; R, rat. Chemical group: AA, aromatic amino/nitro-type chemicals; Alk, natural electrophiles, including reactive halogens; Misc, minor groups of structurally alerting chemicals; inert halogen, nonalerting chemicals containing a nonreactive halogen; minor structural concerns, nonalerting chemicals but with minor concerns; no structural alerts, chemicals devoid of actual or potentially electrophilic centers. Adapted from Ashby and Tennant (*30*).

each other. This means that classes or groups of carcinogens that are not identified by one test should be preferentially identified by another. There was a lack of complementarity among the four assays: these assays, for the most part, identified the same carcinogens. Any combination of the assays led to an increased number of carcinogens correctly identified, but at the cost of an equal number of noncarcinogens giving positive results, the so-called false positives. It was therefore concluded that no combination of tests improved the predictivity of the Salmonella test alone. The high degree of agreement among the tests indicates that all of the assay systems tend to detect similar chemical properties, i.e., mutagenicity, but that the assays other than SAL, to varying degrees, appear to respond positively to other chemical properties that are not directly related to the capacity of the chemicals to induce cancer.

These results have been fortified by an examination of chemical substructure in relationship to carcinogenicity (*29–32*). The results presented in Figure 1 are derived from an analysis and evaluation of 301 chemicals that have been tested for carcinogenicity in two rodent species and for mutagenicity in Salmonella (*30*). The evaluation demonstrated some important aspects of these chemical structure–activity relationships. First, it is possible to group the chemicals that are mutagenic in Salmonella into three broad structural categories. The three structural categories that include the majority of mutagens are shown in the legend of Figure 1 and include aromatic amines, chemicals with alkylating functions, and a heterogeneous group of chemicals that possess halogen atoms generally substituted into a reactive position in the molecule. The fact that it has been possible to categorize virtually all of the 301 chemicals among six broad structural groupings indicates also that the selection of chemicals for testing in rodent bioassays over the past two decades has not been a completely random process. Thus, this selection of chemicals is not necessarily representative of the universe of chemicals that are present in the environment, used in commerce and medicine, etc. These chemicals represent those that were selected for testing to fulfill the needs of the different government agencies or those for which there was some evidence or suspicion that they might possess carcinogenic potential.

Figure 1 also stratifies the chemicals into six levels of biological effect, which reflects their activity in the 2-year rodent carcinogenicity studies. One measure of the relative biological potency of the chemical is the extent to which it is able to cross species or to induce tumors at multiple versus single sites. These results show that a higher proportion of mutagenic carcinogens, as defined by mutagenesis in Salmonella, induced trans-species carcinogenic effects than did nonmutagens. Nonmutagens, on the other hand, included an increased proportion of chemicals that induced single-site and single sex–species carcinogenic effects (see below).

Studies by others suggested that it may be possible to identify some proportion of the nonmutagenic carcinogens by determining whether they have the capacity to induce genetic toxicity in the whole animal. The assays most often used to estimate this are the induction of micronuclei, chromosome aberrations, or sister chromatid exchanges in rodent bone marrow cells. Our evaluations of these associations are not yet completed, but the results suggest that the carcinogenic mutagens often induce chromosomal aberrations or micronuclei. Therefore, these systems may supplement the data obtained *in vitro* or by observation of chemical structure. However, there are also some dichotomies in the data that indicate that the induction of chromosomal damage *in vivo* alone will not be sufficient to adequately discriminate between all carcinogens and noncarcinogens. Later in this article we discuss the potential application of mutagenesis systems in transgenic mice toward this problem.

An interesting association has been identified by Shelby and Zeiger (*33*) among the chemicals identified as group 1 human carcinogens by the International Agency for Research on Cancer (IARC). With very few exceptions, the organic substances represented among these agents are also mutagenic in Salmonella, induce micronuclei in rodent bone marrow cells, and possess structural alerts as defined by Ashby and Tennant (*30*). Taken together, these results show that mutagenic chemicals have a high probability of inducing carcinogenic effects in multiple species and that these properties are also demonstrated by the majority of chemicals that have been shown to cause human cancers. Thus, mutagenicity, as reflected both in chemical structure and biological activity, can be used as a primary risk factor for identifying chemicals with trans-species carcinogenic potential. Although a significant number of chemicals that have the capacity to induce cancers following long-term exposures in rodents fail to show mutagenicity in Salmonella and/or for structural alerts, this does not diminish the positive value of mutagenicity assays.

Represented in the result shown in Figure 1 are 62 mutagens that have failed to demonstrate carcinogenic potential in both rats and mice (levels E and F). Many of these chemicals are structurally similar to mutagenic carcinogens. One plausible hypothesis is that this fraction of mutagens, under the conditions tested, did not gain effective access to the cellular genome, even upon chronic exposure of rodents. This may be because such chemicals are effectively metabolized or detoxified or are not distributed to appropriate target tissues in which they can interact to induce neoplastic change. There may be modifying chemical substructures associated with such chemicals that in some way reduce the capacity for electrophilic interactions, or there may be other factors that are not yet understood.

Another interesting aspect to the data shown in Figure 1 is that the great majority of mutagenic chemicals that were not carcinogens when tested in both rats and mice occurred in bioassays that were conducted and evaluated during the 1970s. Of the chemicals placed on test by the National Cancer Institute or the NTP up through 1980, approximately 31% of the noncarcinogens and equivocal carcinogens were mutagenic in Salmonella; this value was 10% for those chemicals placed on test after 1981. These results may indicate that there have been changes in the selection of chemicals, that criteria and methods of postmortem examination and identification of tumors has changed over this period of time, or that the criteria for determining carcinogenicity have changed. However, these "false positives" do not diminish the positive value of mutagenicity for identifying potential carcinogens. The association appears so high that it can be argued that chemicals with biological and structural evidence of mutagenic potential should be tested only to determine if they are not carcinogenic, or in order to classify the relative biological potential of the mutagens. Mutagens demonstrating trans-species potential represent a clear potential hazard for human health.

Although the data derived from the Salmonella assay are highly informative about mutagenic potential, there does not appear to be any relationship between rodent carcinogenicity and the doses at which the chemical induces mutations, the number or types of Salmonella strains in which mutations are induced, or the shapes of the dose–response curves. That is, a positive

response in any of the Salmonella strains is informative about mutagenic and carcinogenic potential, but none of the other parameters improve our understanding of the relative potency of the chemical, either for its mutagenicity in other systems or for the ability of the chemical to induce carcinogenic effects in rodents.

Within the NTP, the identification of potential mutagens is based on an examination of the primary structure of the chemical and testing using *in vitro* and *in vivo* short-term tests. A positive response in any of the tests is based on both statistical and empirical considerations and requires the independent verification of the original results, i.e., a repeatable positive or negative response in a completely independent assay. All of the tests are conducted under code without knowledge of the structure of the chemical and many of its physical properties. This imposes limitations on the identification of some types of potential mutagens where problems are encountered involving solubility, reactivity with plastic, high toxicity, etc., factors that are often difficult to accommodate in standardized protocols. Therefore, it has been necessary to evolve protocol modifications to ensure that substances such as those that are highly volatile or may require different types of metabolism can be adequately tested. Under some circumstances the chemical may be studied for its ability to induce either micronuclei in peripheral blood cells in mice or for its ability to induce micronuclei or chromosomal aberrations in bone marrow cells of rats or mice. Chemicals that subsequently demonstrate a positive response in both the Salmonella and the *in vivo* cytogenetics assays are considered to be confirmed *in vivo* mutagens and will be predicted to have a high probability of inducing a carcinogenic effect in the 2-year bioassay. Conversely, if a chemical is mutagenic in Salmonella, a negative response in an *in vivo* genetic toxicity test does not diminish the implication of the positive Salmonella response for carcinogenesis.

Nonmutagenic Carcinogens

In 1973, Ames and his co-workers (*2*) declared that carcinogens are mutagens. However, the analysis of the mutagenicity results from 114 chemicals studied for carcinogeniity by the NTP, has shown that approximately 50% of chemicals that are carcinogenic in rodent bioassays are not mutagens (*28,34*) and also lack evidence of structural features consistent with electrophilic potential (*29,30*). In comparison, only 48% of the carcinogens in the same 114-chemical dataset were carcinogenic in both rodent species, and the overall concordance between mice and rats for carcinogenicity and noncarcinogenicity was 69% (*35*).

The nonmutagenic carcinogens have been a focal point of discussion and controversy because they have led to presumptions about the inadequacy of genetic toxicity assays for predicting rodent carcinogens. As stated previously, mutagenicity assays identify chemicals that have the capacity to interact with DNA or other macromolecules either directly or following metabolism. There are chemicals that lack such capacity for direct interaction that nonetheless may be genotoxic by virtue of their ability to indirectly affect DNA replication or repair or chromosome metabolism or to affect other pathways that can give rise to heritable mutations. There has been speculation that the indirect induction of mutations could be an important mechanism. For example, some chemicals, by virtue of effects on cell homeostasis or metabolism, by inducing inflammation, or by inhibiting repair processes, could increase the amount of damage to DNA resulting from an increase in oxidative radical production (*36–38*). Various oxygen radicals are produced as a by-product of normal cellular metabolism, and a variety of processes normally exist to sequester such products and prevent them from causing injury in the cell. In addition, there are repair mechanisms to guard against inadvertent DNA damage. It is possible that some chemicals could alter any one of these complex pathways, and the failure of such protection systems could give rise indirectly to an increase in mutations.

The patterns of carcinogenicity induced by nongenotoxic chemicals are also of interest. As described previously, a proportion of mutagenic chemicals produced tumors at specific sites and often induced tumors across species and at multiple sites. In contrast, a high proportion of the single-species, single-site carcinogens are not mutagenic and do not contain structural alerts. The results imply that both the processes and consequences of carcinogenicity associated with exposure to nonmutagens are fundamentally different from those resulting from exposure to many mutagenic chemicals. Ames and Gold (*36*) have proposed that nonmutagenic carcinogenesis in 2-year rodent bioassays is likely to be a consequence only of the toxic effects of chemicals or their capacity for effects on indirect induction of mutations. We have recently analyzed the relationships between the subchronic and chronic nonneoplastic effects of 31 chemicals that have been tested for carcinogenicity in 2-year rodent bioassays (*39*). The results of this study did not reveal any consistent association between the capacity of chemicals to induce chronic (nonneoplastic) toxicity and subsequent tumor induction. Rather, there were examples where both mutagenic and nonmutagenic chemicals induced toxic effects, many of which showed proliferative characteristics such as hyperplasia, that did not result in tumor induction. Mutagenic chemicals, by and large, tend to be more toxic than do nonmutagens (*35*), but there were sites of tumorigenesis by both mutagens and nonmutagens that were associated with toxicity and others that were not. These results failed to support a direct relationship between sustained tissue-specific toxicity and tumorigenic potential. The results imply that properties of chemicals other than their ability to induce either mutations or specific tissue toxicity may be important in the induction or promotion of carcinogenic processes.

The difficulty in trying to identify the nongenotoxic chemicals that have carcinogenic potential represents a major problem. Among such chemicals represented in the NTP database are a diversity of structural and physical groupings, and it has not been possible thus far to identify any particular features that have predictive value. A significant number of nonmutagenic substances were included among the 44 chemicals that were used to predict potential carcinogenicity, as described below. For these chemicals, the only animal information available that might relate to their carcinogenic potential was the patterns of subchronic toxicity that they induced in the two rodent species. In an effort to challenge the relationships between such toxicity and tumorigenicity, the available evidence was used in efforts to predict whether such chemicals would be carcinogenic. When these results are evaluated they will provide an additional test of the association between toxicity and carcinogenicity. Thus, the problem of predicting carcinogenic potential remains. The strategy that is currently being used by the Experimental Carcinogenesis and Mutagenesis Branch (ECMB) of NIEHS is to seek a better understanding of

the mechanisms by which such chemicals may act, rather than to randomly seek methods that may have some predictive capacity. If some common mechanisms or biological effects can be identified for many nonmutagenic carcinogens, they would lead to a rational and logical basis for developing short-term test systems that would provide useful information for predicting carcinogenicity.

Mechanisms of Carcinogenicity of Nonmutagenic Carcinogens

There have been a variety of mechanisms proposed to account for the carcinogenicity of nonmutagenic chemicals. Prominent among these ideas is the induction of oxidative damage. A particular example of this mechanism is the ability of chemicals to induce the proliferation of peroxisomes, which may be a significant source of mutagenic oxygen radicals. Thus, the proposed mechanisms for many chemicals classified as "nonmutagens" may involve some form of indirect mutagenesis. The evidence that links the induction of somatic mutations with the genesis of cancer is compelling. Several lines of evidence strongly support the association. They include the identification of specific chromosome damage in many rodent and human tumors (*40*), the identification of oncogenes that have been activated by specific mutations (*41*), or the inactivation of tumor-suppressor genes also by mutation (*42*), and, of course, the mutagenicity of known human carcinogens (*43*). The scientific momentum created by these lines of evidence support the multistage concept of carcinogenesis. There is also the recent finding of a series of gene and chromosomal mutations in human colorectal cancers (*44*). In addition, the somatic mutation hypothesis is consistent with experimental two-stage models of initiation and promotion.

Although these lines of evidence are compelling, there are also other lines of evidence that support alternative mechanisms of carcinogenesis. As a result of the NTP, and other studies, we now know that there are more differences among the mutagenic and nonmutagenic carcinogens than simply their mutagenicity in Salmonella (*28,45*), supporting the existence of alternative mechanisms of carcinogenesis. These alternative mechanisms include the induction of cell proliferation, induction of peroxisomes, changes in methylation patterns, changes in gene expression, activation of oncogenes, etc. This is also an area of investigation where chemical structure–activity relationships may be useful. Among the evidence supporting these alternative mechanisms is the frequently observed regression or remodeling of neoplasms and the existence of noninduced spontaneous tumors in rodents that have a genetic component in their expression.

The major problem with the concept of nonmutagenic carcinogenesis is how to account for the heritable phenotypic change that must accompany the evolution of neoplasms and how to explain the genetic changes that are associated with many tumors. It has been proposed that many of the processes that give rise to cancer cells represent analogs of the changes that occur during normal growth and differentiation. For example, heritable patterns of gene expression are responsible for the emergence of differentiated cells from stem cell populations, for patterns of terminal differentiation and apoptosis, and for the maintenance of differentiated characteristics of organs and tissues.

Relatively little is known about how patterns of gene expression are heritably altered. The methylation of cytosine bases has been implicated in some types of transcriptional regulatory mechanisms, but this clearly does not represent the only means by which gene expression is controlled. The association of histone proteins can also affect the capacity of genes to be transcribed, but beyond these mechanisms the complex associations among transcriptional factors, polymerases, and transcribable genes is yet to be defined. We believe that the transcriptional apparatus is a plausible target for heritable changes induced by chemicals. The adaptive capacity of mammalian cells in culture is well demonstrated. Cells removed from the highly regulated and controlled environment of the body are plated onto plastic surfaces in the presence of foreign serum, and cell populations with the capacity for extended growth emerge. This same adaptive capacity might be expressed *in vivo* in the presence of sustained toxicity. For example, in the 2-year rodent bioassays, although efforts are made to limit the level of toxicity to a maximum-tolerated dose, the consequences of long-term exposure often cannot be anticipated from subchronic toxicity studies. The animals are thus inadvertently exposed to levels of chemicals that bring about various types of organ-specific toxicity. In order for cells to survive in the presence of toxicity, a variety of changes in gene expression occur, and in order for daughter cells to survive in the presence of sustained toxicity, they too must express the same altered pattern of gene expression. It is plausible that sustained, altered gene regulation, where proliferation is also driven by toxic effects, can create situations in which further alterations in genomic regulation occur and could be a substrate from which chromosome and other genetic alterations emerge. Farber and Rubin (*46*) have described a similar selective process that they call stepwise adaptation, which appears to be a plausible mechanism of epigenetic or nongenotoxic carcinogenesis. The challenge for the future is to better identify the phenomenon of nongenotoxic carcinogenesis and define experimental systems in which these hypotheses can be tested.

Prediction of Carcinogenicity

The rodent carcinogenesis bioassay presents a unique opportunity for assay validation because chemicals that are subjected to the testing are not definitively known to be either carcinogenic or noncarcinogenic. Thus, the information generated from an *in vitro* assay system, and other information about the chemicals' structure and chemical and biological properties, can be used to predict the potential results of a rodent bioassay in a prospective approach to validation. We are currently testing the relationships among chemical structure, Salmonella mutagenicity, and carcinogenicity that are advocated in this paper. In this effort, 44 chemicals that were in some phase of the rodent bioassay, but for which the results of the assay were not yet known, were selected only on the basis that they were under assay within a particular time frame, i.e, 1990–1992. Data on the structure and short-term biological properties of the chemicals were compiled and used for predicting their carcinogenic potential. These predictions were published in *Mutagenesis* (*47*), and the editor of *Mutagenesis* invited other groups with different predictive methodologies to also submit for publication their predictions on these same chemicals. Six additional manuscripts were published, predicting at least a portion of the 44 chemicals. When the majority of the bioassays are completed in 1993, there will be an unprecedented opportunity to challenge the hypothesis of the relationship between mutagenicity and carcinogenicity, as well

as of the ability of various predictive strategies, including computer-assisted methods.

Future assays both for subchronic toxicity and for carcinogenicity will provide the opportunity to challenge new hypotheses, as well as new methods of predicting carcinogenicity. The evaluation of all of these methods are, of course, predicated on the assumption that the rodent bioassay is an unequivocal, or at least the best available, method for carcinogen identification against which all other methods should be measured. This issue has been subject to extensive debate, but most arguments defer to the fact that, despite the uncertainties and limitations of 2-year rodent studies, there are as yet no other reliable methods by which carcinogenic potential can routinely be assessed. Thus, for the foreseeable future, all alternative methods of identifying carcinogens will be measured against the results of 2-year rodent carcinogenicity tests.

We advocate the use of predictive toxicology for the development of other methodologies. However, if the carcinogenesis bioassays are used as experimental tools for testing alternative methods, the process of validation can be better defined, and the period of evaluation can be tightly circumscribed. For example, the ECMB is attempting to assess the relationship between chemical substructure and organ-specific toxicity. The extensive database established by the NTP on the subchronic toxicity of chemicals is even larger than that available for long-term carcinogenicity results and provides an unparalleled source of information on organ-specific toxicity. The assays have been conducted in a systematic manner that allows comparisons within and among assays and chemicals. Various computer-assisted methodologies are currently being evaluated to determine if specific chemical substructures can be identified that are associated with organ-specific toxicity. The identification of such structural alerts can then be experimentally challenged by using this information to predict the outcome of future subchronic toxicity assays. This concept can be used to assess any alternative methodologies purported to be able to substitute for long-term animal studies. But it is only the extensive and well-developed database that has been created from animal studies that makes this approach possible.

Transgenic Cell and Mouse Models

The development of methods to introduce foreign genes into cultured cells or to inject foreign genes into the pronuclei of zygotes is an important advance in genetic methodologies. Application of such methods has resulted in the development of transgenic cultured cells and mouse lines that carry stable integrated copies of foreign genes that can be targeted for expression in certain tissues or under specific environmental conditions and which provide unique targets to study chemical–biological interactions. Model systems that are being generated with these technologies will provide important tools in both carcinogenesis and genetic toxicology. Among the most important models developed thus far that have applications in both fields are mouse lines that carry inserted bacterial genes that can be recovered, in which mutations can be detected and quantitated and for which mutational spectra can be determined. The two most prominent transgenic mouse lines currently being investigated involve the introduction of bacterial *lac*I or *lac*Z genes, which are carried as stable multigene tandems. The target genes can be subsequently recovered using packaging extracts of bacteriophage, plated onto sensitive *E. coli* strains, and the induced mutations quantitated. These mice offer the opportunity for the first qualitative as well as quantitative measurements of chemical-induced, tissue-specific, somatic mutations in mice, and will allow a determination of whether correlations exist between sites of carcinogenesis and mutagenesis induced by carcinogens. They will also provide important models with which to study the problem of indirect or oxidative mutagenesis. Preliminary studies are underway, but within the next few years significant amounts of information will accrue about the tissue-specific mutagenic potential of a variety of chemicals. The ability to recover mutated DNA and to determine mutational spectra provides a method by which the action of specific chemicals can be fingerprinted.

A second application of transgenic methodology is the evaluation of mice carrying oncogenes or proto-oncogenes that act as additional targets for potential carcinogens. These systems will provide the opportunity to assess new methods that may permit a reduction in the use of rodents in 2-year bioassays for carcinogen identification. They will also provide the opportunity to study chemical–gene interactions and the role of chemicals at various stages in the processes of neoplasia in ways in which it has not been possible to conceive of before the development of such models. The ECMB has initiated preliminary studies of the effects of chemicals in three transgenic mouse lines that carry oncogenes under the control of mouse mammary tumor virus (MMTV) regulatory sequences. The three oncogenes v-*Ha-ras*, c-*myc*, and c-*neu* are thus all regulated by glucocorticoid-sensitive MMTV-LT-R elements. The predominant expression of the transgenes is directed to mammary tissue and, to a lesser extent, other sensitive tissues. These three transgenic lines are being studied for their responses to selected chemicals. This is designed to determine whether carcinogens can specifically accelerate or alter the pattern of tumor induction and thus provide useful models for short-term detection of carcinogens under conditions in which chemicals can be metabolized, detoxified, and distributed within the body by normal pathways.

A third line of research involves the use of cultured mammalian cells into which foreign genes can be introduced by either transfection or transduction. The latter method uses selected retroviral vectors, which provide a more efficient means of introducing foreign genes with more complicated constructs. This methodology is being used to determine the effects of selected proto- or activated oncogenes on the growth properties and chemical responsiveness of cultured cells. A particular problem to which these studies is directed is to understand better the differential responses to chemical carcinogens among rodent and human cells. Rodent cells generally adapt to growth in cell culture, readily acquire extended life spans, and respond to a variety of chemical carcinogens that induce morphological and malignant transformation. Human cells, on the other hand, do not readily undergo changes to extended life span upon growth in culture and tend to maintain a finite life span and a high degree of genome stability. Unless they contain specific oncogenes, human cells are also generally unresponsive to the effects of chemical carcinogens. An important issue to be addressed is the biological basis of the relative resistance of human cells. The approach currently being studied is to introduce selected genes into normal human fibroblasts and to determine the effects on growth characteristics and chemical sensitivity.

Conclusions

The efforts of the past two decades serve to reaffirm the complexity of the carcinogenic process. The anticipated simple relationship between mutagenicity and carcinogenicity was not borne out by results from the genetic toxicity and rodent carcinogenicity assays. The complexity of chemicals, together with the complexity of the organisms, the numbers of gene products and genes with which the various moieties of the chemical can interact, and the roles of duration, route, and exposure dose, all contribute to the overall complexity. *In vitro* methodologies and bioassays are biological tools for helping us to understand the nature of chemical properties. The results have clearly delineated two general classes of carcinogens: those that are mutagenic *in vitro* and contain chemical moieties capable of reacting as electrophiles, and those that are not. The mutagenic chemicals support a mechanism of carcinogen induction that is amenable to study by specific cellular, biochemical, and genetic techniques. The nonmutagenic carcinogens encompass a variety of chemical structures and biological responses. There is no reason to believe that this group of carcinogens is effective through a unified mechanism. Some chemicals are believed to be active through the intracellular generation of oxygen radicals, others are believed to act by first inducing cell proliferation, and others through the disruption of normal cell regulatory effects or cell-cycle kinetics.

The nonmutagenic carcinogens, therefore, present a challenge to identify and characterize the cellular mechanisms that are capable of initiating or promoting the carcinogenic response. The subsequent relationships among results obtained from these *in vitro* and rodent systems and the induction or expression of cancers in humans remains another step to be addressed. The results described in this paper outline information upon which future efforts to seek answers to these questions can be based.

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